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A synthetic peptide of the N-terminus of ADP-ribosylation factor (ARF) inhibits regulated exocytosis in adrenal chromaffin cells

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We have investigated the role of ADP-ribosylation factor (ARF) in regulated exocytosis in digitonin-permeabilized adrenal chromaffin cells by the use of a synthetic peptide, hARF1(2–17), based on the N-terminus of the protein. hARF1(2–17) inhibited Ca^{2+}-dependent but not basal exocytosis, whereas equimolar levels of other synthetic peptides were ineffective. The inhibitory effect of hARF1(2–17) was dose-dependent and half-maximal at 12 μM. GTPγS-induced secretion in the presence of non-stimulatory CA^{2+} concentrations was also inhibited by hARF1(2–17). These results point to a hitherto unsuspected role for ARF in regulated exocytosis, and the potency of the hARF1(2–17) peptide suggests that ARF is essential for exocytosis in bovine adrenal chromaffin cells.

Exocytosis; Calcium; ADP-ribosylation factor; GTP-binding protein; Chromaffin cell

1. INTRODUCTION

Regulated exocytotic fusion of secretory vesicles with the plasma membrane is triggered by Ca^{2+} in many cell types [1,2] but the mechanisms involved are not known. The use of permeabilised cells has begun to identify the proteins that may be involved in exocytosis [2] but more detailed characterisation has been achieved for the proteins involved in various other vesicular transport steps [3]. Certain key proteins appear to function in many intracellular membrane fusion events and may also be involved in regulated exocytosis [4]. GTP-binding proteins appear to control all vesicular transport steps in the exocytic and endocytic pathways [5–7]. ADP-ribosylation factor (ARF) [8], a monomeric GTP-binding protein, is a component of the non-clathrin coats of Golgi transport vesicles [9], regulates coat assembly [10] and has been implicated in multiple vesicle transport/fusion steps [11–14]. The N-terminus of ARF is essential for its function and a synthetic N-terminal ARF peptide inhibits ARF function, endoplasmic reticulum (ER)-to- Golgi transport, intra-Golgi transport and endocytic vesicle fusion [10–13]. We have examined the effect of synthetic hARF1(2–17) and found that it inhibits its Ca^{2+}- and GTPγS-dependent secretion from permeabilised adrenal chromaffin cells whereas other peptides are ineffective. These results implicate ARF as a key component of the regulated exocytotic machinery and emphasise the similarity of the mechanisms involved in the vesicular fusion events throughout the secretory pathway.

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2. MATERIALS AND METHODS

2.1. *Materials*

High-purity digitonin was obtained from Novabiochem (Nottingham, UK). Fetal calf serum and Dulbecco's modified Eagle's medium with 25 mM HEPES were obtained from Gibco (Paisley, UK). GTPγS was obtained from Boehringer-Mannheim (Lewes, UK). The rab3AL(33–47) peptide was synthesised in the Department of Biochemistry, University of Liverpool. hARF1(2–17), sec4(1–15), cal(1–15) and SynA(217–231) peptides were synthesized by Multiple Peptide Systems (San Diego, CA, USA), characterised by mass spectrometry and purified by reverse-phase HPLC. All other chemicals were from Sigma (Poole, UK).

2.2. *Isolation and culture of chromaffin cells*

Chromaffin cells were isolated from bovine adrenal medulla by enzymic digestion [15], washed in Ca^{2+}-free Kreb's–Ringer buffer, (145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose and 20 mM HEPES at pH 7.4), resuspended in culture medium (Dulbecco's modified Eagle's medium with 25 mM HEPES, 10% fetal calf serum, 8 μM fluorodeoxyuridine, 50 μg/ml gentamycin, 10 μM cytosine arabinofuranoside, 2.5 μg/ml amphotericin B, 25 U/ml penicillin, 25 μg/ml streptomycin), plated in 24-well trays at a density of 1×10^6 cells per well, and maintained in culture for 3–5 days before use [15].

2.3. *Cell permeabilisation and assay of catecholamine secretion*

After initial washing of cells in Ca^{2+}-free Kreb's–Ringer buffer, the protocol was in two stages unless indicated otherwise. (1) Cells were permeabilized with 300 μl of buffer A (139 mM potassium glutamate, 2 mM ATP, 2 mM $MgCl_2$, 5 mM EGTA, 20 mM PIPES at pH 6.5) containing 20 μM digitonin for 10 min. (2) Cells were stimulated with 300 μl of buffer A containing the indicated calculated free Ca^{2+} concentrations for 20 min. Synthetic peptides were routinely present in both stages unless indicated otherwise. After stage 2, the buffer was removed, centrifuged at $16{,}000 \times g$ for 2 min and aliquots taken for assay of released endogenous catecholamine. Total catecholamine

V S A L G I D F K V K T I Y R N	rab3AL(33-47)
G N I F A N L F K G L F G K K E	hARF1(2-17)
S T V H E I L C K L S L E G D	Cal(1-15)
M S G L R T V S A S S G N G K	Sec4(1-15)
A M L V E S Q G E M I D R I E	SynA(217-231)

Fig. 1. Sequences of the synthetic peptides used in this study. The peptides are all N-terminal amides.

content of the cells was determined after release of catecholamines with 1% Triton X-100. Catecholamine secretion was calculated as a percentage of total cellular catecholamine. All experiments were performed at 22–25°C.

3. RESULTS

We examined the effect of a range of peptides on Ca^{2+}-dependent secretion of catecholamine from digitonin-permeabilised adrenal chromaffin cells. These peptides (Fig. 1) correspond to the putative effector domain of rab3 (rab3AL(33–47) [16], the essential N-terminal domain of human ARF1 (hARF1(2–17) [11]), the N-terminus of annexin II (cal(1–15)), and yeast sec 4 (sec4(1–15)), and a cytoplasmic sequence of the synaptic plasma membrane proteins the syntaxins (synA(217–231) [17] which has homology to related proteins involved in vesicular traffic including the yeast protein SED5 [18]. Although all five peptides were used at a concentration of 100 μM, only hARF1(2–17) inhibited 10 μM Ca^{2+}-induced secretion (Fig. 2). The inhibitory

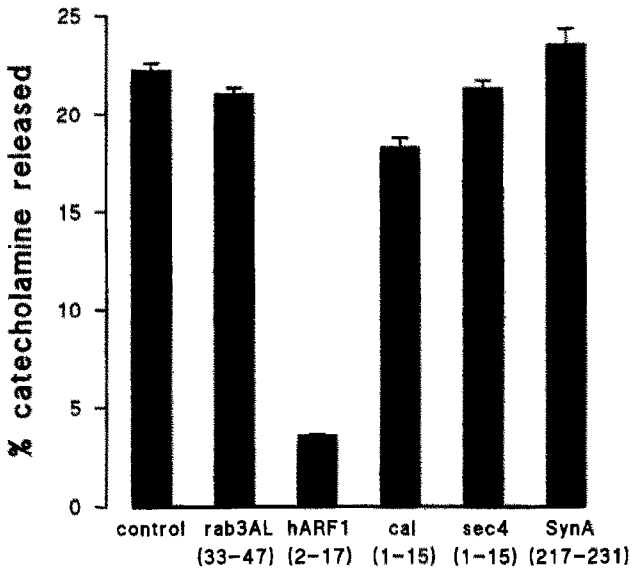

Fig. 2. Effect of various synthetic peptides on Ca^{2+}-dependent secretion from permeabilised adrenal chromaffin cells. Cells were permeabilised with digitonin for 10 min in the absence or presence of 100 μM peptide prior to stimulation with 10 μM Ca^{2+} in the absence or presence of peptide. Catecholamine release over 20 min was expressed as a percentage of total cellular catecholamine. Data shown are mean ± S.E.M. (n = 6).

effect of hARF1(2–17) was reproducible and, in a series of experiments, this peptide inhibited secretion by 83.2 ± 2.5%(n = 9). The rab3AL peptide provides a good control for the examination of the effects of hARF1(2–17). The two peptides contain similar numbers of hydrophobic residues and have the same charge density (three basic and one acidic amino acids) and distribution of charged residues, but rab3AL(33–47) had no effect on secretion in our experiments.

The dose–response for inhibition of secretion by hARF1(2–17) is shown in Fig. 3. This peptide had no effect on basal (Ca^{2+}-independent) secretion at doses up to 50 μM, but inhibited 10 μM Ca^{2+}-induced secretion. Half-maximal inhibition occurred at 12 μM and the effect saturated at around 50 μM. The peptide was therefore effective at similar concentrations to those that inhibit ER-to-Golgi and intra-Golgi transport [11,12], endosome–endosome fusion [13] and β-COP binding to Golgi membranes [10]. At higher doses (> 100 μM) hARF1(2–17) began to stimulate low levels of Ca^{2+}-independent release of catecholamine, and this may reflect a membrane-perturbing effect of high doses of the peptide. The inhibitory effect of hARF1(2–17) on vesicular fusion is believed to be due to tight binding of the peptide to a putative ARF receptor which prevents recruitment of cytosolic ARF to membranes [11–13]. The inhibitory action of hARF1(2–17) on ER-to-Golgi transport was found to be irreversible [12] and we also found that the hARF1(2–27) inhibition could not be reversed by subsequently washing the cells for 15 min prior to stimulation. Again, as reported for ER-to-Golgi transport [12], we found that the inhibitory effect of hARF1(2–17) was partially antagonised when the cells were pre-incubated with the peptide in the presence of brain cytosol. In the experiments shown, hARF1(2–17) was included during permeabilisation but in other experiments it was found to be equally effective when incorporated in a post-permeabilisation step prior to stimulation, ruling out an artefact due to interference with permeabilisation. The peptide inhibition of secretion was most marked when the cells were pre-incubated with the peptide prior to stimulation with Ca^{2+}, and peptide addition with the Ca^{2+} stimulus reduced secretion by only 42%, suggesting that hARF1(2–17) generated a time-dependent block of exocytosis.

Secretion due to micromolar Ca^{2+} in permeabilised chromaffin cells is inhibited by pre-incubation with

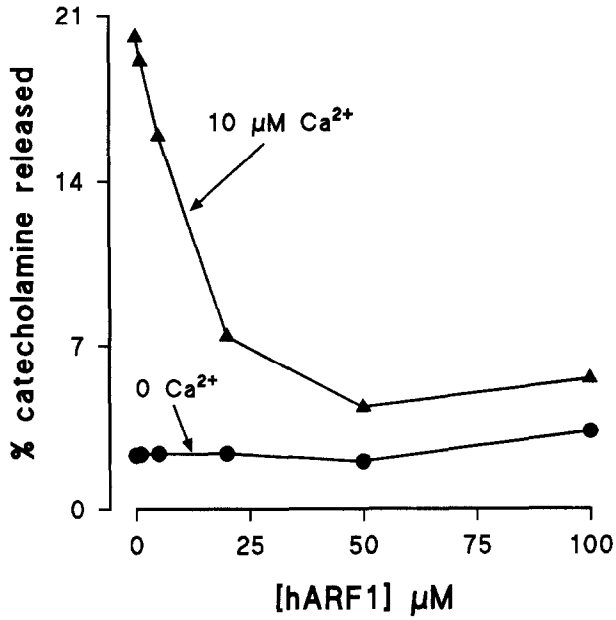

Fig. 3. Dose-dependency of the inhibitory effect of hARF1(2–17). Cells were permeabilised for 10 min in the presence of varying concentrations of hARF1(2–17) prior to incubation with 0 or 10 μM free Ca^{2+} in the presence of hARF1(2–27). Catecholamine released over 20 min was expressed as a percentage of total cellular catecholamine. Data shown are means (n = 6); the S.E.M. was smaller than the symbols and is not shown.

GTPγS [19,20], but at non-stimulatory Ca^{2+} concentrations (300 nM or less) non-hydrolysable GTP analogues stimulate exocytosis [21,22]. As shown in Fig. 4, hARF1(2–17) inhibited not only Ca^{2+}-induced but also GTPγS-induced exocytosis in chromaffin cells, indicating that the peptide inhibits a step required by both types of stimuli.

4. DISCUSSION

N-Terminal ARF peptides have proved to be potent inhibitors of ARF function [10–13]. The specific inhibition of regulated exocytosis in chromaffin cells by hARF1(2–17) implicates ARF as a key part of the exocytotic machinery. A small component of Ca^{2+}-dependent exocytosis was uninhibited by the peptide. The magnitude of this component was similar to that of the cytosol-independent component remaining after prolonged permeabilisation, and this may represent the population of already docked or 'primed' secretory vesicles that exists in chromaffin cells [2,23,24]. Data from work on the effects of GTP analogues on exocytosis in the squid giant synapse has suggested a role for monomeric GTP-binding proteins in vesicle docking prior to Ca^{2+}-activated neurotransmitter release [7]. Recent work has established that ARF proteins are important mediators of vesicular transport, and the results have been interpreted on the basis of a putative role in transport vesicle coat assembly/disassembly [9,11,25]. This is difficult to reconcile with the present data (since chromaffin granules are not coated) and with the observations on ARF involvement in endosome–endosome fusion [13]. The drug, brefeldin A, inhibits various steps in the secretory pathway by effect on coat assembly due to an inhibition of guanine nucleotide exchange on ARF [26,27]. We found no effect of pre-incubation with brefeldin A on Ca^{2+}-dependent secretion in permeabilised chromaffin cells nor did it influence the effects of GTPγS or hARF1(2–17) (data not shown). Therefore, it seems more likely that ARF proteins control membrane targeting and/or fusion, possibly by promoting the assembly of a multi-subunit complex required at the fusion site. This is an attractive hypothesis since numerous cytosolic and membrane proteins have been implicated in fusion in the secretory pathway, and these presumably interact to form a fusion machine [2,3].

The rab family of monomeric GTP-binding proteins also appear to be involved at all stages of the secretory pathway [28]. Synthetic peptides based on the putative effector domain of rab proteins, such as rab3AL (33–47), inhibit ER-to-Golgi and intra-Golgi transport [15] and can stimulate exocytosis [29–31]. Recent work has shown that rab3AL (33–47) had no effect on Ca^{2+}-de-

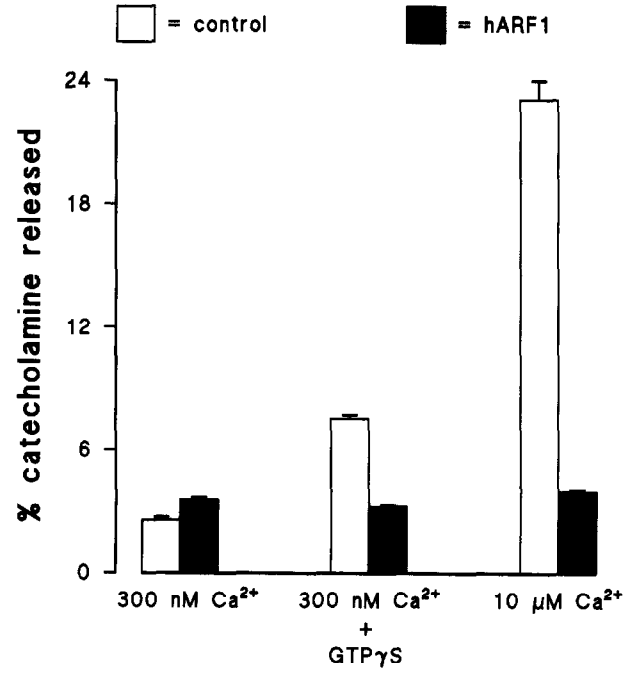

Fig. 4. Effect of hARF1(2–17) on GTPγS- and Ca^{2+}-induced secretion. Cells were permeabilised for 10 min in buffer A containing 20 μM digitonin in the presence or absence of 100 μM hARF1(2–17) and in the presence or absence of 100 μM GTP[S] prior to stimulation with Buffer A containing 300nM free Ca^{2+}, 300nM free Ca^{2+} plus 100 μM GTPγS or 10 μM free Ca^{2+} in the presence or absence of 100 μM hARF1(2–17). Catecholamine released over a 20 min period was expressed as a percentage of total cellular catecholamine. Data shown are means ± S.E.M. (n = 6).

pendent exocytosis in pituitary cells, consistent with data here, but that a longer peptide, rab3AL (30–47), potently inhibited secretion [32]. Thus it appears that both ARF and rab proteins are required for regulated exocytosis. These findings and other recent discoveries [4,33] suggest that the targeting and fusion machinery in regulated exocytosis involves members of the same protein families that act in other intracellular membrane fusion events.

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